HEPATOPROTECTIVE EFFECT OF AZADIRACHTA INDICA (NEEM) IN ALCOHOL-INDUCED LIVER DAMAGE

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ABSTRACT

Alcohol induced liver cirrhosis is a worldwide problem but effective drugs are not available in the market. Many studies reported the patoprotective effect of neem leaves, but no scientific study in alcohol induced liver damage. So the main objective of the present study was to evaluate the protective effect of methanolic extract of Azadirachta indica (A. indica extract) leaves in alcohol induced hepatotoxicity in wistar rats. Hepatotoxicity was produced by administering graded doses of ethanol orally for 4 weeks. A. indica extract were given to treated groups at a dose of 200 mg/kg and 400 mg/kg orally for 4 weeks. Similarly Silymarin 100 mg/kg were given to standard group rats. Biochemical parameters (ALP, SGPT, SGOT, TP and S. Bilirubin), liver weight and histopathological parameters were evaluated. A indica extract (both doses) significantly prevented the changes in the above biochemical parameters. Similarly it significantly prevented the elevation of liver weight and histological changes as compared to the ethanol treated group. The above results suggest that Azadirachta indica is useful in treating alcohol induced liver damage.

Key Words: Azadirachta indica, ethanol induced liver injury, ALP, SGPT, SGOT, histopathology
INTRODUCTION
Liver disease is still a worldwide health problem. Hepatotoxic drugs like ethanol (widely consumed worldwide) can injure the hepatocyte directly via a free radical and metabolic intermediate that causes peroxidation of membrane lipids and that result in liver cell injury.[1] Earlier reports suggested that chronic alcohol ingestion elevates the cytokine level (TNF-alpha, IL-1 and IL-6) in hepatic cells which leads to liver injury and this action of alcohol is quite different from the mode of action of other hepatotoxicants.[2] Unfortunately, conventional or synthetic drugs used in the treatment of alcohol induced liver disease are inadequate and sometimes can have serious side effects.[3] Indigenous drugs possessing fewer side effects are a better alternative for the treatment of liver diseases.

Azadirachta indica (Neem tree) is such an indigenous plant growing throughout India possessing wide range of medicinal properties. Neem leaf consists of several valuable components and can be divided into two major classes: isoprenoids that include terpenoids containing limonoids, azadirone and its derivatives, c-secomeliacins and non-isoprenoids include amino acids, polysaccharides, sulphurous compounds, polyphenolics like flavonoids and their glycosides.[4, 5] Extensive literature review revealed that neem leaves are effective in curing carbon tetra chloride,[6] paracetamol,[7] anti-tubercular drug[8] and mercuric chloride induced liver damage.[9] But there are no studies reported on the hepatoprotective effect of neem leaves in alcohol induced liver damage.

In view of these considerations neem leaves which already showed its hepatoprotective effect in other models were selected for the current study. The aim of the study was to evaluate the hepatoprotective activity of methanolic extract of Azadirachta indica leaves in chronic alcohol-induced liver injury.

MATERIALS AND METHODS
Drugs and chemicals
ALP assay kit (Dr. Reddy’s Laboratory), strips of SGPT, SGOT, TP and S. Bilirubin (Idexx), Silymarin (Sigma) and all other reagents used in the study were of analytical grade.

Plant material collection and Identification
Fresh leaves of Azadirachta indica were collected in the month of April from the plain areas in Guwahati, Assam, India. The plant material was authentified in the Department of Botany, Gauhahi University, Guwahati.
Preparation of plant extract
The leaves were washed with water and shade dried in open air, then pulverized to dry powder using an electric grinder. About 500 gm of the powder was extracted with 4 litres of methyl alcohol (70%) by cold maceration for 7 days. The extract was filtered, evaporated using vacuum rotary evaporator (Buchi) and heated on a water bath at 45 ± 5°C to obtain A. indica extract (12.17% yield w/w). Carboxy methyl cellulose (0.5%) was used as solvent to prepare different doses of A. indica extract.

Animals
Wistar albino rats of either sex (150-200gm) were used for the present investigation and were obtained from the animal house of Gauhati Medical College, Guwahati. Animals were housed under standard environmental conditions of temperature (25±2°C) and light & dark cycle (12:12 h). Rats were fed with standard pellet diet and water ad libitum. All experimental studies were done after getting permission from the Institutional Animal Ethics Committee, Gauhati Medical College, Guwahati.

Preliminary phytochemical investigation
Preliminary phytochemical screening of A. indica extract was performed using standard procedures. Test for flavonoids: 1ml of the extract was mixed with dilute NaOH; golden yellow precipitate confirmed the presence of flavonoids. Test for saponins: 1ml of the extract was mixed with 10ml of warm distilled water; formation of persistent foam indicated the presence of saponins. Test for glycosides: 1 ml of extract was mixed with 1 ml of water and 5-6 drops of 10% sodium hydroxide solution; a yellow colour confirms the presence of glycosides. Test for tannins: 1 ml of extract was mixed with 1 ml of 10% lead acetate solution; a white precipitate confirms the presence of tannins.

Acute dose toxicity study
The acute toxicity study was carried out as per the OECD guidelines 425. Initially A. indica extract was administered orally at a limit dose of 2000 mg/kg to a single female rat. The rat was observed closely for the first 4 hr and then periodically up to 24 hr for any toxic symptoms and mortality. After 24 hr same dose was administered to four more female rats.
Evaluation of hepatoprotective activity of A. indica extracts

Wistar albino rats were divided into 5 groups and each group contains 6 animals.\textsuperscript{[16]}

1. Group 1: Negative control (0.5% CMC 10 ml/kg p.o.)
2. Group 2: Positive control (10\% ethanol (v/v) in water for 1 week, 20\% (v/v) for another 1 week then 30\% (v/v) for the next 2 weeks of the experiment)
3. Group 3: Standard control (Silymarin 100 mg/kg p.o + 10\% ethanol (v/v) in water for 1 week, 20\% (v/v) for another 1 week then 30\% (v/v) for the next 2 weeks of the experiment)
4. Group 4: Test 1 (A. indica extract 200 mg/kg p.o. + 10\% ethanol (v/v) in water for 1 week, 20\% (v/v) for another 1 week then 30\% (v/v) for the next 2 weeks of the experiment)
5. Group 5: Test 2 (A. indica extract 400 mg/kg p.o. + 10\% ethanol (v/v) in water for 1 week, 20\% (v/v) for another 1 week then 30\% (v/v) for the next 2 weeks of the experiment)

All the above treatments were given for 4 weeks. On the 29\textsuperscript{th} day, blood was collected from the rats by retro orbital plexure method. Serum was separated from the collected blood, and used for the estimation of alkaline phosphatase (ALP), serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), Total protein (TP) and serum bilirubin. ALP was estimated by using the assay kit provided by Dr. Reddy’s Laboratory and rest of the parameters were estimated by automated vet analyser (IDEXX) using the respective strips supplied along with the analyser.\textsuperscript{[17]}

After collection of blood samples rats were sacrificed humanely. Liver was dissected out and cleared off of the surrounding tissues. The weight of individual liver was noted and kept in 10\% buffered neutral formalin, dehydrated in alcohol, and then embedded in paraffin. The paraffin blocks were sectioned at a size of 5-\mu m and stained with haematoxylin and eosin for histological examinations.\textsuperscript{[18]}

Statistical analysis

Values were expressed as mean ± SEM. Statistical analysis was performed using one way ANOVA (Graph pad prism version 6) followed by Dunnett's post hoc test and values of P<0.05 were considered to be statistically significant.\textsuperscript{[19, 20]}
RESULTS
Preliminary phytochemical investigation
Preliminary Phytochemical investigation of *A. indica* extract showed the presence of glycosides, tannins, flavonoids and saponins.

Acute dose toxicity study
*A. indica* extract did not show any toxic reactions and mortality up to a dose of 2000 mg/kg. So LD$_{50}$ of *A. indica* extract should be more than 2000 mg/kg. For the current research work 200 mg/kg and 400 mg/kg were taken as treatment dose.

Evaluation of hepatoprotective activity of *A. indica* extract
The results of hepatoprotective activity of *A. indica* extract on alcohol treated rats are shown in Figure 1-6. The hepatic enzymes (ALP, SGPT and SGOT), liver weight and bilirubin in serum were increased in alcohol treated rats when compared to control. Moreover, in positive control rats TP was reduced when compared to negative control group. The *A. indica* extract treatments significantly reversed the levels of ALP (P<0.001), SGPT (P<0.001), SGOT (P<0.001) and S. Bilirubin (P < 0.001) when compared to positive control rats.

![Figure 1: The effects of *A. indica* extract in Serum ALP level in Wistar rats](image)

Values are expressed as mean± SEM (n=6). *p*<0.001 as compared to positive control group.
Figure 2: The effects of *A. indica* extract in SGPT level in Wistar rats
Values are expressed as mean± SEM (n=6).  a  p<0.001 as compared to positive control group.

Figure 3: The effects of *A. indica* extract in SGOT level in Wistar rats
Values are expressed as mean± SEM (n=6).  a  p<0.001 as compared to positive control group.

Figure 4: The effects of *A. indica* extract in TP level in Wistar rats
Values are expressed as mean± SEM (n=6).  a  p<0.001,  c  p<0.05 as compared to positive control group.
Liver weight (P < 0.05) and TP level (P < 0.001) were also normalised by *A. indica* extract treatments when compared to positive control rats. Silymarin 100 mg/kg was also showed similar results.

Histological examination of alcohol treated rats showed evidences of liver damage (fatty changes, chronic inflammatory cells, central vein congestion and necrotised hepatocytes). Rats treated with *A. indica* extract 200 mg/kg showed a partial degeneration of liver tissue. But Silymarin 100 mg/kg and *A. indica* extract 400 mg/kg treated rats showed completely regenerated liver cells.
Figure 7: The effects of *A. indica* extract in liver histopathology in Wistar rats

Histology of liver stained with Haematoxylin-Eosin (×100): A) Negative control B) Positive control C) *A. indica* extract 200 mg/kg D) *A. indica* extract 400 mg/kg E) Silymarin 100 mg/kg.

DISCUSSION

Ethanol is widely used as a hepatotoxin in experimental studies. The efficacy of any hepatoprotective drug is essentially dependent on its capacity of either reducing the harmful effects or maintaining the normal hepatic physiology which is disturbed by the hepatotoxin.\[21\] Ethanol accumulation in liver cells leads to production of xanthine oxidase which generates reactive oxygen species such as superoxide anion and hydrogen peroxide.\[22\] The Kupffer and the endothelial cells of the liver as well as the hepatocytes contain xanthine dehydrogenase that is readily converted into xanthine oxidase.\[23\] Ethanol produces hypoxia in liver cells triggers conversion of xanthine dehydrogenase into xanthine oxidase.\[24\] Oxygen
free radicals could be involved in triggering liver injury by increasing transcription factors such as NF-kB, which induce inflammatory cytokine productions (TNF-alpha, IL-1 and IL-6).

In liver injury enzymes were leaked into blood because of enhanced permeability, injury and necrosis of hepatocytes. Hence SGPT, SGOT and ALP levels are elevating in liver toxicity.[25] In the present study, A. indica extract significantly reduced the SGPT, SGOT and ALP level in serum of treated group is a clear indication of hepatoprotective effect. Hepatocyte cytoplasm is very rich in bilirubin and in cell damage bilirubin is abundantly leaked in to blood.[26] A reduction of S. bilirubin in the current study confirms the hepatoprotective activity of A. indica extract.

Another characteristic feature of hepatic damage observed is significant decrease in serum protein level in ethanol treated rats. A. indica extract treatment for 4 weeks significantly increased the total protein level further confirms the hepato protective effect of neem extract. Moreover the increase in liver weight showed by alcohol treated rats was also cured by A. indica extract treatment. Chronic alcohol treatment increases the liver weight by blocking the secretion of hepatic triglycerides in to blood.[27] Histopathological results further added more proofs to the hepatoprotective effect of A. indica extract.

Preliminary phytochemical evaluation revealed the presence of glycosides, tannins, flavonoids and saponins in the A. indica extract. It have been reported that flavonoids, tannins and saponins shows promising hepatoprotective activity.[28] Acute dose acute toxicity studies failed to reveal any toxic effect of A. indica extract and LD$_{50}$ should be more than 2000 mg/kg of body weight.

**CONCLUSION**

From the present investigation it is evident that, the methanolic extract of Azadirachta indica leaves possessed significant hepatoprotective activity against ethanol intoxication in rats. Hence apart from earlier studies the current study showed that neem leaves can reduce the chronic alcohol induced liver failure cases which are rapidly elevating currently. However, it needs isolation, structural elucidation, and screening of any of the above mentioned active principle/s to establish the clinical utility.
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REFERENCES


